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DRUG DISCOVERY

A simple RP-HPLC method for Acyclovir determination in tablet and cream dosage forms

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ABSTRACT

A simple, rapid, accurate, and precise reverse phase HPLC (RP-HPLC) method was developed and validated for the determination of acyclovir (ACV) present in the pharmaceutical dosage form. Chromatographic separation was carried out on an asymmetric® C18 column (4.6×75 mm) packed with 3.5-μm spherical particles. The mobile phase consisted of water: acetonitrile (95:5 v/v) flowing at a rate of 1.9mL/min; the column temperature was 30°C, and a photodiode array detector was used. The runtime under these chromatographic conditions was 3.0 min. The chromatogram showed a peak of ACV at the retention time of 0.77 ± 0.12 min, with no interfering peaks. The method was linear over the range 2–20 μg/mL. The proposed method was further applied to the determination of ACV in the pharmaceutical dosage form (i.e., as tablet or cream), as well as in the determination of drug contents with good percentage recoveries. The accuracy and precision of the method were validated on an intraday and interday basis in accordance with the ICH guidelines.

Keywords: Acyclovir; Liquid chromatography; RP-HPLC; Validation; Pharmaceutical dosage forms; Tablets; Cream

1. INTRODUCTION

Acyclovir (ACV), 9-[(2-hydroxyethoxy)-methyl]methyl]-guanosine, is an acyclic guanosine derivative that selective inhibits the replication of herpes viruses, with potent clinical antiviral activity against the herpes simplex and varicella zoster viruses (Schaeffer et al., 1978). Acyclovir determination in pharmaceuticals has been performed spectrophotometrically. The visible spectrophotometric method, which is not very sensitive (Basavaiah and Prameela, 2002, Ayad et al., 2007), a derivative spectrophotometric (Daabees, 1998, Sultan, 2003) technique, and a differential spectrophotometric method (Mahrous et al., 1992) have all been proposed.

Several HPLC RP methods for ACV (Huidobro et al., 2005, Tzanavaras and Themelis, 2007, Caamano et al., 1999) have been used to determine the concentration of the drug in serum (Bahrami et al., 2005, Cronqvist and Nilsson-Ehle, 1988) and in the pharmaceutical dosage forms as oral suspensions (Palma-Aguirre et al., 2007, Basavaiah et al., 2003), tablet dosage form (Palma-Aguirre et al., 2007, Patil et al., 2014, Basavaiah et al., 2003), capsule dosage form (Tu et al., 2001), mucoadhesive dosage form (Tao et al., 2009), microsphere dosage form (Stulzer et al., 2008), and topical cream dosage form (Krishnaiah et al., 2014, Inoue et al., 2013).

A new sensitive HPLC, a simple and fast method for ACV determination with acetaminophen as the IS, was developed in this study and further used for the quantitative determination of ACV concentrations in different dosage forms (tablets and creams), and utilized for drug content analysis. Additionally this method is characterized by a short run time.

Although there is no structural similarity between ACV (Boulieu et al., 1997) and acetaminophen (Abu-Qare and Abou-Donia, 2001) (Figure 1), they have similarities in their solubility behaviours in the mobile phase used and detected at the same wavelength, which makes acetaminophen an appropriate choice as the IS for this study.

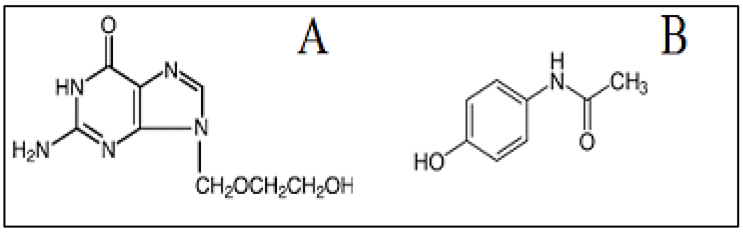

Figure 1 Chemical structure of acyclovir (A; drug) and acetaminophen (B; IS)

2. MATERIALS AND METHODS

Reagents and chemicals

Acyclovir and acetaminophen, the IS, were gifted by Spimaco (Riyadh, Saudi Arabia). All the other reagents and chemicals were of HPLC analytical grade, and were used as received. Water was deionized and purified by a Milli-Q Reagent Grade system (Millipore Corporation, Bedford, MX, USA).

Table 1 The acyclovir brands tested in this study

Symbol	Trade name	Dosage from	Strength	Manufactured by
T1	Zovirax®	tablet	200 mg	Glaxo Wellcome. Aranda, Spain. Packed by Glaxo Saudi Arabia Ltd. SA
T2	Virustat®	tablet	200 mg	Ram Pharmaceutical Industries Co. Ltd, Jordan
C1	Zovirax®	cream	5% (10 gm)	Manufactured by: Glaxo Wellcome. Aranda, Spain . Packed by Glaxo Saudi Arabia Ltd. SA
C2	virol®	cream	5% (10 gm)	Riyadh Pharma, Riyadh, Saudi Arabia

The different dosage forms of ACV included tablets and creams. All the tests were performed before the expiry dates. Two different 200-mg ACV tablets were used in the present study: the original product, ACV-A (GlaxoSmith Kline K.K.), and the following one, a generic product: ACV-B (Ram Pharmaceutical Industries Co. Ltd, Jordan). The two products were randomly named ACV-T1 and ACV-T2 (Table 1). Two different 5% ACV creams were used in the present study: the original product, ACV-A (Glaxo Smith Kline K.K.), and the following one (a generic product): ACV-B (Riyadh Pharma, Riyadh, Saudi Arabia). The two products were randomly named ACV-C1 and ACV-C2 (Table 1).

Chromatographic system and conditions

A new sensitive method for ACV was developed in this study. It involved a simple procedure suitable for routine work. The concentration of ACV was measured using a Waters HPLC system equipped with a Waters 2707 auto sample and 2998 photodiode array detector. A Waters 1525 solvent delivery system was used to operate the isocratic flow through a symmetric® C18 column (4.6 x75 mm) packed with 3.5 μm spherical particles. The mobile phase consisted of water: acetonitrile (95:5 v/v). The mobile phase was prepared daily during the study. Degassing was achieved by filtration through a 0.22-μm Millipore membrane filter and sonication for 10 min. The injected volume was 10 μL. The mobile phase flow rate was 1.9 mL/min, and the run time was 3.0 min. Data were collected with a Breeze Chromatography Manager Data Collection System. The HPLC system was operated at 30°C. A daily standard calibration curve (six standards ranging from 2 to 20 μg/mL) was performed to determine the unknown ACV concentration.

Preparation of standard solutions and calibration curve

A stock solution of concentration 50 μg/ml was prepared by dissolving 5.0 g of ACV, accurately weighed, in 10.0 ml of 0.1 N sodium hydroxide in a 100-ml volumetric flask, and stirred in an ultrasonic bath for 10 min. The solution was made up to 100 ml with methanol. As the stock solution for the IS, 100 mg of acetaminophen was dissolved in 25 ml of methanol and stored at -20°C. For calibration, different standard working solutions of ACV (2–20 μg/mL) and the IS (100 μg/mL) were prepared by diluting the abovementioned stock solutions in pure methanol and were kept at -20°C. The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs.

Sample preparation of the pharmaceutical dosage forms

Sample preparation of the pharmaceutical dosage forms or the analysis of tablet dosage form, five tablets were weighed individually and their average mass was determined. The tablets were then crushed to a fine powder. An accurately weighed portion (~10 mg) of the powder was dissolved in 10 mL of methanol in a 200-mL volumetric flask and was stirred in an ultrasonic bath for 15 min to extract the drug from the microspheres. The volume was made up with the mobile phase. 5 mL of this solution was diluted with the mobile phase in a 50-mL volumetric flask to obtain a concentration of 5 μg/mL. The sample used for the specificity assay was prepared in the same form, injected into the chromatographic system, and analyzed quantitatively.

For the analysis of cream dosage form, the method reported by Tzanavaras and Themelis (Tzanavaras and Themelis, 2007) was adapted herein. Accurately weighed amounts of the pharmaceutical creams (three 100-mg samples of Zovirax® and Virol® creams), were dispersed in 50.0 mL of 0.01M NaOH. The samples were stirred ultrasonically for another 30 min until complete dissolution of the cream. 5-mL portions of the resulting suspensions were filtered through disposable syringe filters (0.45 m pore size, Whatman) and were injected in triplicate into the HPLC system for determining acyclovir concentration.

Assay Validation

The developed method was validated in terms of linearity, precision, accuracy, and robustness, according to ICH Harmonized Tripartite Guidelines (Guideline, 2005).

Assessment of Linearity, Accuracy, Precision and Robustness

Three standard calibration curves were prepared at different times (over a period of at least three months) to evaluate the linearity, precision, accuracy, and stability.

The linearity of each standard curve was assessed by plotting the peak area ratio of ACV-to-IS versus ACV concentration. Accuracy of the process was determined in term of bias (percentage deviation from the nominal concentration= [100*(actual concentration-nominal concentration)/ actual concentration]. The accuracy was assessed by examining multiple replicates (n = 6) of ACV concentration.

Precision of a measurable technique is the degree of agreement among individual tests, when the technique is applied repetitively to analyze multiple replicates on three different occasions. The intraday precision was assessed by analyzing the calibration curves of six replicates with different ACV concentrations within the same day. The inter-day precision was determined by the analysis of six replicates with different ACV concentrations on three different days. The total precision of the method was expressed in terms of the relative standard deviation (RSD%).

Low-, medium-, and high-concentration quality control (QC) samples at ACV concentrations of 20, 120, and 200 μg/mL, with 100μg/mL of IS, were analyzed on three different occasions within a period of at least three months, as described above. The limits of detection (LOD) and quantification (LOQ) were measured based on the analysis of 6 replicates. LOD and LOQ for ACV were

determined based on the signal-to-noise concept, as the lowest concentrations at which the signal-to-noise ratiosare 3:1 and 10:1, respectively. The robustness of an analytical procedure is the measure of its capacity to remain unaffected by small, but deliberate, variations in method parameters and provides an indication of its reliability during normal usage. The robustness was studied by evaluating the effect of small deliberate variations in the chromatographic conditions.

Data and statistical analysis

In-vitro results were expressed as the mean ± SD values of at least three replicates. The HPLC results of ACV were calculated using linear regression without weighting, according to the equation: Y= 0.0403x − 0.0119, where Y is the ratio of the area under the peak (AUP) values of the drug to the internal standard and X is the ACV concentration. The RSD% was calculated for all the values. The student's *t*-test was used to inspect the concentration difference on each day and one-way analysis of variance (ANOVA) was used to assess the reproducibility of the assay and the drug dissolution from batch to batch using IBMSPSS Statistics 21. The level of confidence was 95%.

3. RESULTS AND DISCUSSION

The proposed method was optimized to develop an analytical method for effective determination of ACV. Figure 2 shows the HPLC chromatogram A for the blank, mobile phase, and chromatogram B for ACV and IS with average retention times of 0.77 ± 0.12 and 2.15 ± 0.15 min, respectively, with no interfering peaks. This is an indication the specificity of the HPLC assay method. Reported HPLC-based methods for ACV determination need longer time for detection of ACV alone, or operate without any IS.

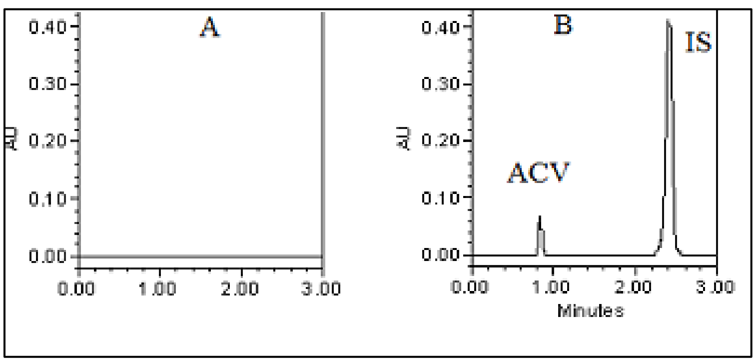

Figure 2 HPLC chromatograms of blank mobile phase (chromatogram A) and HPLC chromatograms of mobile phase containing 2 µg/mLACV and 100 µg/mL acetaminophen as IS (chromatogram B).

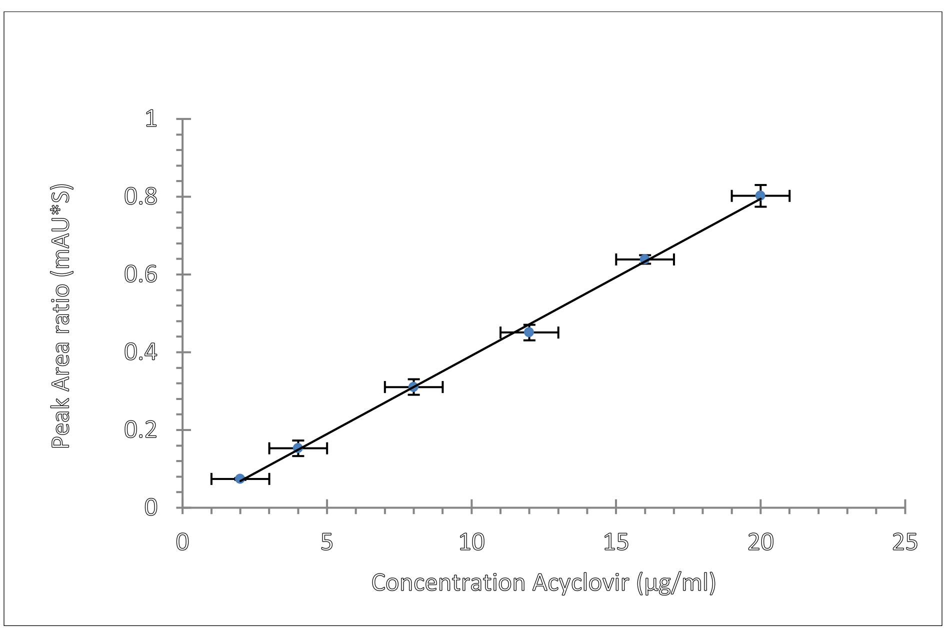

Figure 3 Standard calibration curve of *in-vitro* ACV concentration ± SD in methanol (n = 6)

Figure 3 shows a calibration curve of ACV-to-IS peak area ratios versus concentrations (20–200 µg/mL), as shown in Table 2. Each point on the calibration curve represents a mean of six different determinations. A linear plot was obtained for the range of concentration tested with a correlation coefficient (*r*) of 0.9989. The regression equation was Y= 0.0041x − 0.017. Therefore, a good linear relationship was observed between the ACV peak area ratios and its tested concentrations.

Table 2 Acyclovir mean calibration curve (n=6)

µg/mL	2	4	8	12	16	20
PAR-1	0.072	0.15	0.298	0.456	0.633	0.801
PAR-2	0.074	0.163196	0.337958	0.471	0.623	0.784
PAR-3	0.069	0.142712	0.317958	0.449	0.58	0.836
Average	0.072	0.152	0.318	0.459	0.612	0.807
SD	0.002517	0.010383	0.019979004	0.01123981	0.02816	0.026514
RSD%	3.511551	6.832318	6.283258802	2.450540015	4.601349	3.28552

PAR: Peak area ratio; SD: Standard deviation; RSD%: Relative standard deviation.

The closeness or similarity among different measurements in a series of measurements, derived from multiple sampling of the same homogenous solution under similar conditions, is an indication of assay precision. The interday and intraday precision values are measures of the method variability that can be expected when used for practical purposes (Shaikh et al., 2008) and is expressed in terms of RSD%. The intraday precision (Table 3) was determined by analyzing six replicates each with ACV concentrations of 20, 120, and 200 µg/mL. For interday precision determination (Table 4), six replicates each with concentrations of ACV were analyzed on three different days. The relative standard deviations were determined. For determination of accuracy, the relative error was calculated. The RSD% values obtained were between 1.07 and3.57 for intraday precision and between 1.09 and 4.34 for interday precision, indicating good precision for the developed method.

Table 3 Intraday precision determination (n = 6)

Concentration (µg/mL)	2	12	20
Average	0.072	0.46	0.80
SD	0.002	0.01	0.008
RSD%	3.57	2.4	1.07

Table 4 Interday precision determination (n = 6)

Concentration (µg/mL)	20	120	200
Average	0.07	0.46	0.80
SD	0.003	0.01	0.008
RSD%	4.34	2.89	1.09

The accuracy, calculated as the relative error of the average concentrations determined by HPLC compared to standard ACV solutions, was determined at low, medium, and high concentrations (i.e. 20, 120 and 200 µg/mL, respectively) of ACV solution. The RSD% values were between 3.05 and 10.58 for intraday accuracy (Table 5) and between 3.54 and 5.16 for interday accuracy (Table 6), indicating that the method is sufficiently accurate for determination of ACV in solutions.

Table 5 Intraday accuracy determination (n = 6)

Concentration (µg/mL)	20	120	200
Average	19.67	118	201.67
SD	2.08	3.61	6.66
RSD%	10.59	3.06	3.30

Table 6 Interday accuracy determination (n = 6)

Concentration (μg/mL)	20	120	200
Average	19.87	117.67	202.33
SD	1.03	4.16	10.02
RSD%	5.17	3.54	4.95

The assay method was robust because a small intended change in the assay conditions, e.g. changing the column or the HPLC system, did not have any significant effect on the chromatographic performance of the ACV detection system. Even a little difference in the mobile phase composition did not show any noticeable effect on the peak area of ACV. Two selected available ACV tablets and cream (in the Saudi market) were analyzed by the described HPLC method. In both cases, there should be no interferences according to the suggested method. Some ACV samples were stored away from direct sunlight at 25 °C and a relative humidity (RH) of 84% and some were stored at 4 °C (in a refrigerator) to examine their stabilities. ACV contents in these samples were calculated using HPLC. Results of the ACV assays are shown in Table 7. The ACV contents in all the tablets and creams tested were found to remain at 95–105% for up to 14 days under both sets of storage conditions. Thus, all the formulations complied with the USP guideline (Ueda et al., 2009).

Table 7 Mean ACV contents according to HPLC (±S.D., n = 3)

	Condition	Day-0	Day-7	Day-14
T1	25 °C RH 84%	98.2 ± 0.6	102.2 ± 0.5	99.2 ± 0.9
	4 °C	101.2 ± 0.8	100.8 ± 0.8	102.2 ± 0.6
T2	25 °C RH 84%	103.2 ± 1.1	100.3 ± 1.1	103.5 ± 0.6
	4 °C	99.4 ± 0.7	101.7 ± 0.7	101.1 ± 0.9
C1	25 °C RH 84%	101.6 ± 0.6	98.6 ± 1.3	99.5 ± 0.9
	4 °C	102.2 ± 0.7	99.2 ± 1.5	100.5 ± 0.4
C2	25 °C RH 84%	103.2 ± 0.8	100.3 ± 1.0	101.7 ± 0.8
	4 °C	101.5 ± 0.7	101.7 ± 0.9	103.3 ± 1.1

The developed HPLC assay was applied for the quality control of ACV in both tablet and cream dosage forms by utilizing the accelerated (ACS) and long-term (LTS) stability controls; the results are shown in Table 8, while a representative chromatogram of the tablet and cream, dosage forms are depicted in Figure 4. Long-term stability control of the formulation involves storage at 25 °C/RH 60%, while the corresponding conditions for accelerated stability control are 40 °C/RH 75%.

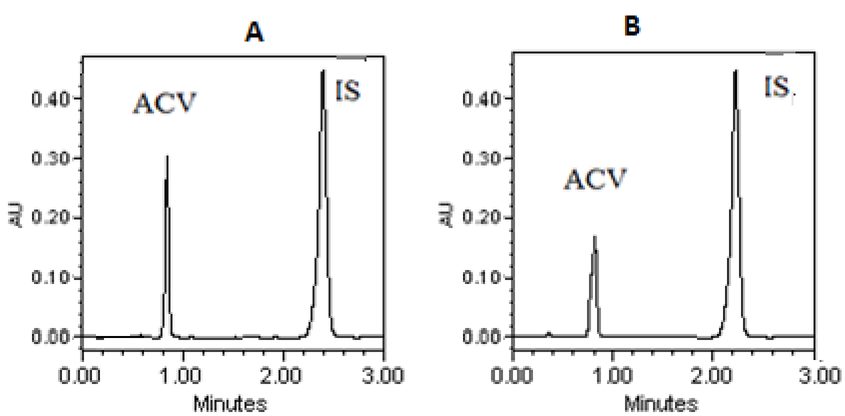

Figure 4 Typical chromatograms of an ACV-containing pharmaceutical tablet (A) and cream (B)

Table 8 Mean ACV contents according to HPLC in different dosage forms (±S.D., n = 3)

sample	Assay (± SD) %					
	0-month		3- month		6- month	
	ACS	LTS	ACS	LTS	ACS	LTS
T1	100.8	100.8	101.1	101.1 (±1.1)	99.5	99.5

	(±0.9)	(±0.9)	(±1.1)		(±1.6)	(±1.6)
T2	99.3 (±1.3)	102.3 (±1.3)	101.1 (±0.7)	103.4 (±0.9)	98.4 (±1.2)	101.5 (±1.1)
C1	102.4 (±1.1)	103.8 (±0.6)	97.3 (±1.4)	102.1 (±1.3)	102.3 (±1.1)	98.5 (±1.6)
C2	98.5 (±1.3)	102.8 (±1.1)	100.2 (±1.0)	103.2 (±0.8)	103.8 (±0.7)	102.5 (±1.6)

ACS: accelerated stability control; LTS: long-term stability control

A simple sensitive, selective, precise, and accurate isocratic assay for ACV was developed and validated. The developed RP-HPLC assay was applied for the quality control of ACV in different dosage forms (tablets and creams) as well as for the assessment of quality control after accelerated and long-term stability control of ACV in these pharmaceutical dosage forms.

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Abbreviations
Acyclovir (ACV)
Reverse phase (RP)
Internal standard (IS)

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Conflict of Interest:
The authors declare that there are no conflicts of interests.

Peer-review:
External peer-review was done through double-blind method.

Data and materials availability:
All data associated with this study are present in the paper.

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